ZOOLOGICAL SCIENCE 11: 451-454 (1994)

Molecular Evolution of Shark C-type Natriuretic Peptides

Masayoshi Takano[1], Yuichi Sasayama[2]
and Yoshio Takei[3]

Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164 and [2]Department of Biology, Faculty of Science, Toyama University, Gofuku, Toyama 930, Japan

ABSTRACT—C-type natriuretic peptides (CNP) of varying length were isolated from the atrium or ventricle of a shark, *Lamna ditropis* and their amino acid sequences were determined. Although the sequence of *Lamna* CNP was highly homologous to those of other CNPs sequenced to date, the *Lamna* CNP-41, the longest CNP identified in this study, has one amino acid replacement from those of *Triakis scyllia* and *Scyliorhinus canicula*, and three amino acid replacements from that of *Squalus acanthias*. The degree of similarity of CNP molecules coincides well with their systematic positions in the cladogram of elasmobranchs; *Lamna, Triakis* and *Scyliorhinus* belong to the same order, but *Lamna* and *Squalus* belong to different orders. The facts that *Lamna* and *Triakis* are in different suborders but *Triakis* and *Scyliorhinus* are in the same suborder and have identical CNP-41, also support this evolutionary implication.

INTRODUCTION

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family first identified in the brain of pig and teleost fishes [2, 4, 9]. In contrast to other members of the peptide family, namely atrial (A-type), B-type and ventricular natriuretic peptides (ANP, BNP and VNP) which are cardiac hormones circulating in the blood, CNP has been isolated from the brain in all species from teleost to mammals, and its plasma and cardiac concentrations are too low to be detected in mammals [8]. Thus CNP is regarded as a neuropeptide in mammals. However, we have isolated CNP from the heart of two species of dogfish shark, *Triakis scyllia* and *Scyliorhinus canicula* [5, 6]. In these fish, plasma and cardiac concentrations of CNP are extremely high, and other cardiac natriuretic peptides, ANP, BNP and VNP, are not identified in their hearts [7]. Furthermore, only CNP cDNA has been cloned from the cDNA library of the heart of spiny dogfish, *Squalus acanthias* [3]. Therefore it is likely that CNP is the only natriuretic peptide present in elasmobranchs. It is also noted that the amino acid sequence of CNP is more conserved than any other natriuretic peptides, namely ANP, BNP and VNP [8]. Thus, CNP might be an ancestral molecule of the natriuretic peptide family, and other members might be reproduced by gene duplication.

As a prototype of the natriuretic peptide family, it seems of interest to examine chemical evolution of the CNP molecule. In previous studies, we have found that amino acid sequences of CNP-22, a mature form stored in the brain, of *Triakis* and *Scyliorhinus* are identical, and even proCNP differs in only 3 out of 115 amino acid residues [5,6]. However, *Squalus* CNP-22 predicted from the cDNA sequence differs from that of *Triakis* in 2 amino acid residues, and the difference was much greater at the level of prohormone [3]. Systematically, *Triakis* and *Scyliorhinus* belong to the same suborder *Scyliorhinoidei*, but *Squalus* is different from the two species at the level of order [1]. We recently have obtained the heart of *Lamna ditropis*. This fish belongs to the order *Lamniformes* as do *Triakis* and *Scyliorhinus*, but to the suborder different from those sharks. Therefore, we attempted in the present study to isolate CNP from the *Lamna* heart and to compare its structure with those of other sharks.

MATERIALS AND METHODS

Isolation of CNP

The shark, *Lamna ditropis*, of approximately 3 m in body length was caught in Toyama Bay and was obtained from fishermen 5 h after capture. The heart was immediately dissected out, the atrium and ventricle separated, and frozen in a deep freezer at −50°C. The atrium (106.4 g) and ventricle (333.2 g) were treated separately. ANP-like peptides in the heart were isolated with protocols described previously [5]. The frozen tissues were crushed in a pulverizer, boiled in 5 volumes (atrium) or 3 volumes (ventricle) of water for 10 min, acidified with AcOH to a concentration of 1 M, and homogenized in a Polytron homogenizer (Kinematika, Germany) for 90 sec at maximum speed. The homogenate was centrifuged at 16,000×g for 30 min at 4°C. The supernatant was added to 2 volumes of cold acetone, and centrifuged at 16,000×g for 30 min at 4°C. The supernatant was evaporated, reconstituted in 30 ml of 1 M AcOH, and added to 2 liters of cold acetone. After centrifugation, the pellet was dissolved in 30 ml of 1 M AcOH, and applied onto a column (5×85 cm) of Sephadex G-25 fine (Pharmacia, Sweden) for desalting. The fractions which contain molecules with Mr>ca. 2,000 were applied onto a column of SP-Sephadex C-25 (1.6×20 cm), and adsorbed materials were eluted successively with 150 ml each of 1 M AcOH, 2 M pyridine, and 2 M pyridine-AcOH, pH 5.0. Each fraction was evaporated and assayed for relaxant activity in the

Accepted June 7, 1994
Received April 4, 1994
[1] Present address: Research Institute, Zenyaku Kogyo Co. Ltd., Ohizumimachi, Nerima, Tokyo 178, Japan
[3] To whom all correspondence should be addressed

chick rectum as described below. Bioactive fractions were subjected to cation-exchange high performance liquid chromatography (HPLC) in an IEC-CM column (7.5×75 mm, Jasco, Japan). Each bioactive fraction was then subjected to reverse-phase HPLC in an ODS-120T column (4.6×250 mm, Tosoh, Japan) with different gradients of CH_3CN concentrations. The detailed chromatographic conditions are described in the legend of each figure. The purified material was subjected to amino acid sequencing in a protein sequencer (477A, Applied Biosystems, USA). Validity of the amino acid sequence was examined by mass spectrometry (JMS-HX110, JEOL, Japan).

ANP-like activity was assayed at each step of purification using a relaxant activity in the chick rectum [10]. New-born male chicks were purchased from Kanagawa Poultry Cooperation (Yokohama) and reared under a infra-red lamp with free access to food and water. The chick was decapitated, rectum immediately isolated, and set up in a trough whose temperature was controlled at 37°C. The rectum was precontracted with 2×10^{-6} M carbachol (Sigma, USA), and the relaxation was quantified by a displacement transducer connected to a transducer amplifier (1829 and 45347, NEC-Sanei, Japan). ANP-like activity was expressed as equivalents to eel ANP which was used as standard.

RESULTS

Same molecules were isolated from atrial and ventricular extracts. After Sephadex G-25 chromatography, fractions of 1–70, which contain molecules larger than CNP-22 [5], were pooled and subjected to SP-Sephadex C-25 chromatography (Fig. 1a). Since only the fraction eluted with pyridine-AcOH exhibited rectum-relaxant activity, this fraction was subjected to cation-exchange HPLC. The bioactive

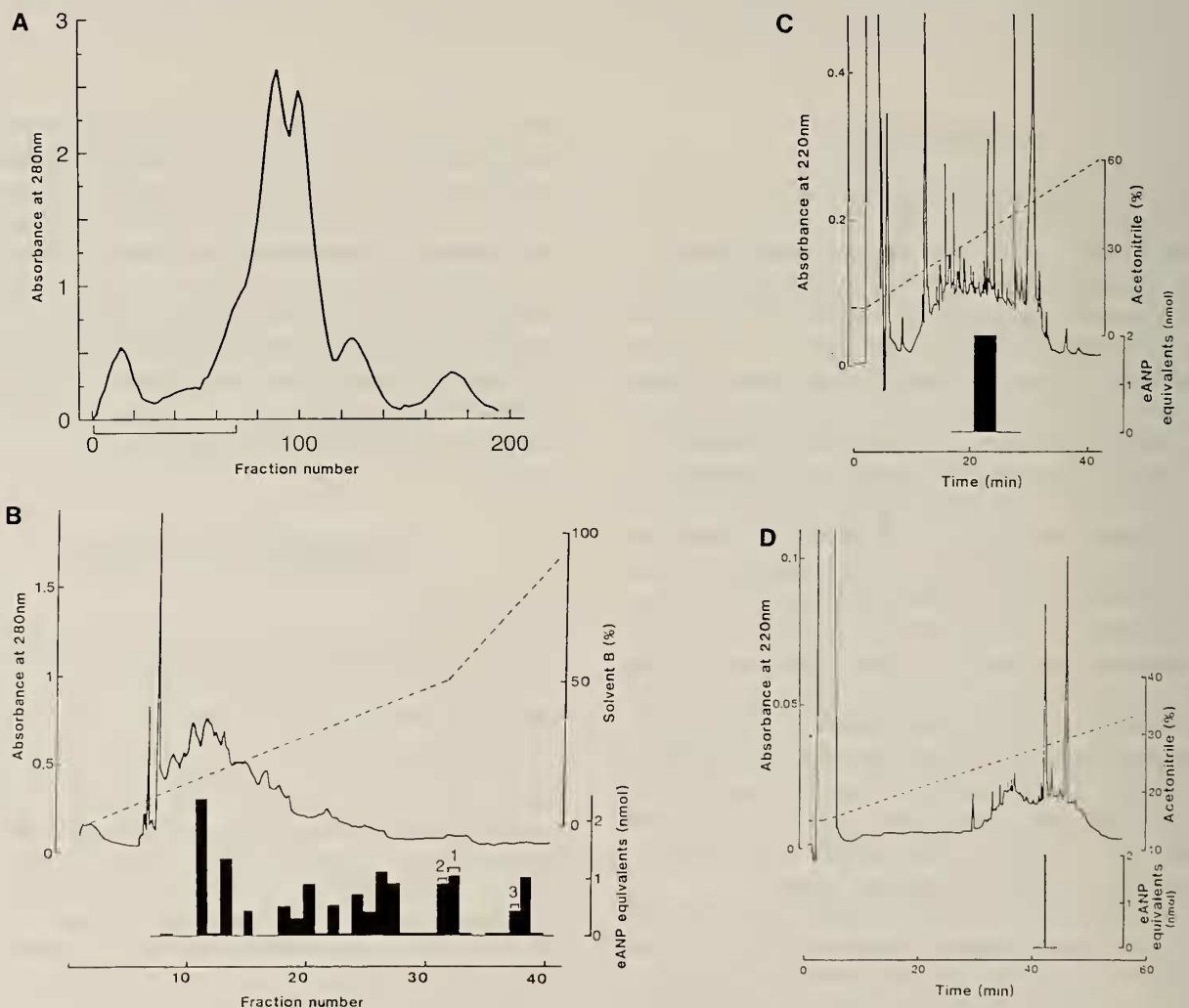

Fig. 1. Purification of C-type natriuretic peptide (CNP) from *Lamna* atrium. Solid columns represent relaxant activity in the chick rectum expressed as equivalents to eel atrial natriuretic peptide (eANP). A: Sephadex G-25 chromatography of crude atrial extract. Fractions marked by square bracket were subjected to SP-Sephadex C-25 chromatography. B: cation-exchange high-performance liquid chromatography (HPLC) of the fraction eluted with pyridine-AcOH in SP-Sephadex C-25 chromatography. Broken lines show gradient of solvent B (1 M NH_4OAc : CH_3CN=9:1) against solvent A (10 mM NH_4OAc : CH_3CN=9:1). CNP-29, CNP-38 and CNP-41 were recovered, respectively, from fractions marked with bracket 1, 2, and 3. C and D: reverse-phase HPLC of fraction 37 of pancl B and a fraction with bioactivity in panel C, respcctively. Sequence analysis of bioactive peak in panel D revealed that the peak is that of CNP-29. Broken lines show gradient of CH_3CN concentrations.

principle was purified only from fractions 31, 32 and 37 of cation-exchange HPLC, although bioactivity was also noted in other fractions (Fig. 1b). A rectum-relaxant principle was isolated from fraction 32 by two steps of reverse-phase HPLC (Figs. 1c, d). Final yield was 2 nmol equivalent to eel ANP as determined by the rectum-relaxant activity and 363 pmol equivalent to eel ANP as determined by absorbance at 220 nm. Sequence analysis of 3/4 of the purified peptide revealed that the sequence was H-Phe-Lys-Gly-Arg-Ser-Lys-Lys-Gly-Pro-Ser-Arg-Gly-(Cys)-Phe-Gly-Val-Lys-Leu-Asp-Arg-Ile-Gly-Ala-Met-Ser-Gly-Leu-Gly-(Cys)-OH (Fig. 2). The presence of two cysteine residues was deduced from the similarity to other CNPs thus far sequenced. Thus the peptide was named *Lamna* CNP-29. The sequence was confirmed by mass spectrometry using the remaining 1/4. A CNP with 12 amino acid residues (Arg-Leu-Leu-Lys-Asp-Leu-Ser-Asn-Asn-Pro-Leu-Arg-) elongated from the N-terminus of CNP-29 was isolated from fraction 37 and thus named *Lamna* CNP-41. The final yield was 260 pmol as judged by absorbance at 220 nm. Sequence analysis of 3/4 of the purified peptide could determine only 28 amino acid residues from the N-terminus. However, it was apparent that the peptide had longer sequence and terminated with the second-half cysteine at the C-terminus, because mass analysis calculated the MH^+ of 4433 which coincides well with the average mass of predicted sequence of CNP-41 (Mr=4432.3). CNP-38 was also isolated from fraction 31 with the final yield of 312 pmol. Although many other fractions showed bioactivity, no bioactive principle could be isolated from those fractions.

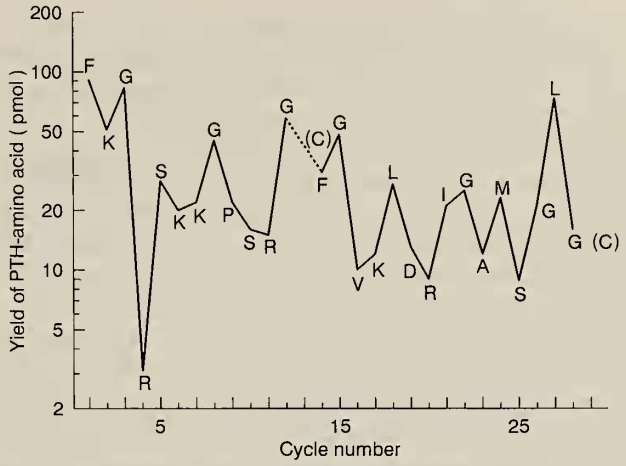

FIG. 2. The yield of phenylthiohydrantoin-derivatized (PTH) amino acid at each cycle of Edman degradation in the sequence analysis of CNP-29. No PTH amino acid was detected at 13th and 29th cycle. The presence of cysteine residues, denoted by (C), was estimated at these cycles from analogy to other CNPs and from the result of mass spectrometry.

DISCUSSION

We isolated three short forms of CNP from the heart of *Lamna ditropis* in the present study. In previous attempts to isolate ANP-like peptides from the heart of other sharks, *Triakis scyllia* and *Scyliorhinus canicula*, large amounts of proCNP and small amounts of CNP-38 and CNP-39 were isolated [5, 6]. CNP-38 was also isolated in this study, but

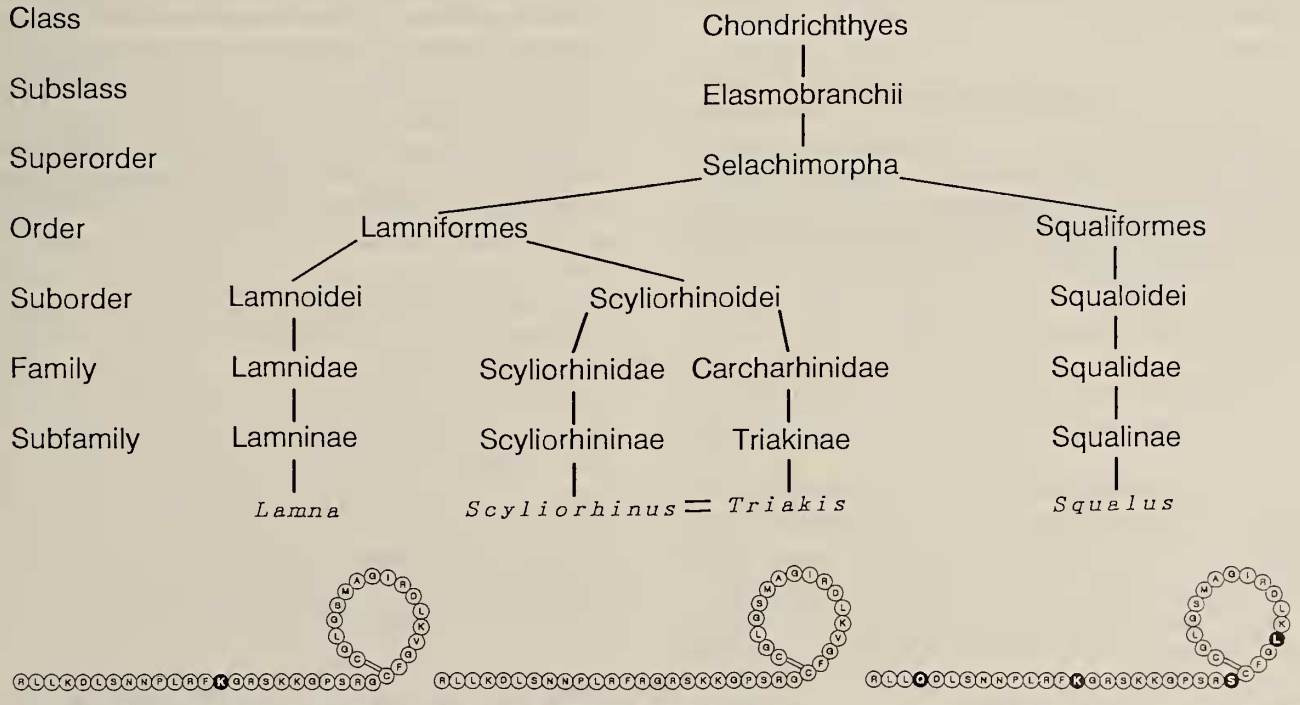

FIG. 3. Phylogenetic cladogram of 4 species of sharks and their amino acid sequences of CNP-41. The cladogram was depicted after Nelson [1]. Amino acid residues different from *Triakis* or *Scyliorhinus* CNP are shaded.

proCNP and CNP-39 were not identified. Instead, CNP-29 and CNP-41 were recovered from the *Lamna* heart. Several peaks with bioactivity, which may contain other fragments of CNP, were also identified after ion-exchange HPLC. This may indicate that a different processing system is operating in the *Lamna* heart, or the shorter forms are degradation products of proCNP. The latter is more likely because it took longer to freeze the *Lamna* heart after its death. In previous studies using *Triakis* and *Scyliorhinus*, hearts were frozen on dry ice immediately after isolation from anesthetized fish.

In addition to *Triakis* and *Scyliorhinus* CNP, CNP cDNA has been cloned from the heart of *Squalus acanthias* [3]. Comparison of the amino acid sequence of CNP-41 between *Triakis* and other sharks revealed that *Triakis* CNP-41 is identical to that of *Scyliorhinus*, is different by one amino acid residue from that of *Lamna*, and is different by four amino acid residues from that of *Squalus* (Fig. 3). As also shown in Figure 3, *Lamna, Triakis* and *Scyliorhinus* belong to the same order (*Lamniformes*) but *Squalus* is in a different order [1]. *Triakis* and *Scyliorhinus* are the same even at the level of suborder, whereas *Triakis* and *Lamna* are in different suborders. It is of interest to note, therefore, that the chemical evolution of CNP molecule is closely related to the cladogram of cartilaginous fishes which is drawn based on the morphological proximity (Fig. 3).

During the course of purification, we utilized relaxant activity in the chick rectum as an assay system. We found that the final yield of CNPs quantified by this assay was always much greater than that deduced from absorbance at 220 nm. It seems therefore that the shark CNP has much greater relaxant activity than eel ANP which was used as standard for the assay.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Hideshi Kobayashi of Zenyaku Kogyo Co. Ltd. for critical reading of the manuscript, and to Dr. T. Takao and Professor Y. Shimonishi of the Institute for Protein Research, Osaka University for performing mass spectrometry. We also thank Dr. T. Sato of Misaki Marine Biological Station, University of Tokyo for his advice on the classification of elasmobranchs and Mr. H. Kaiya and K. Ukawa of Toyama University for their help in the initial part of purification. This investigation was supported in part by grants from the Ministry of Education, Science and Culture of Japan, from the Fisheries Agency of Japan and from Zenyaku Kogyo Co. Ltd.

REFERENCES

1 Nelson JS (1984) Fish of the World. John Willey & Sons, New York, 2nd ed, pp 49–59

2 Price DA, Doble KE, Lee TD, Galli SM, Dunn BM, Parten B, Evans DH (1990) The sequencing, synthesis, and biological actions of an ANP-like peptide isolated from the brain of the killifish, *Fundulus heteroclitus*. Biol Bull 178: 279–285

3 Schofield JP, Jones DSC, Forrest Jr JN (1991) Identification of C-type natriuretic peptide in heart of spiny dogfish shark (*Squalus acanthias*). Am J Physiol 261: F734–F739

4 Sudoh T, Minamino N, Kangawa K, Matsuo H (1990) C-type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. Biochem Biophys Res Commun 168: 863–870

5 Suzuki R, Takahashi A, Hazon N, Takei Y (1991) Isolation of high-molecular-weight C-type natriuretic peptide from the heart of a cartilaginous fish (European dogfish, *Scyliorhinus canicula*). FEBS Lett 282: 321–325

6 Suzuki R, Takahashi A, Takei Y (1992) Different molecular forms of C-type natriuretic peptide isolated from the brain and heart of an elasmobranch, *Triakis scyllia*. J Endocrinol 135: 317–325

7 Suzuki R, Togashi K, Ando K, Takei Y (1994) Distribution and molecular forms of C-type natriuretic peptide in tissues and plasma of an elasmobranch, *Triakis scyllia*. Gen Comp Endocrinol (in press)

8 Takei Y, Balment RJ (1993) Natriuretic factors in non-mammalian vertebrates. In "New Insights in Vertebrate Kidney Function" Ed by JA Brown, RJ Balment, JC Rankin, Cambridge University Press, Cambridge, pp 351–385

9 Takei Y, Takahashi A, Watanabe TX, Nakajima K, Sakakibara S, Takao T, Shimonishi Y (1990) Amino acid sequence and relative biological activity of a natriuretic peptide isolated from eel brain. Biochem Biophys Res Commun 170: 883–891

10 Watanabe TX, Noda Y, Chino N, Nishiuchi Y, Kimura T, Sakakibara S, Imai M (1989) Structure-activity relationships of alpha-human atrial natriuretic peptide. Eur J Pharmacol 147: 49–58

ZOOLOGICAL SCIENCE 11: 455-465 (1994)

Systematic Study on the *Chaenogobius* Species (Family Gobiidae) by Analysis of Allozyme Polymorphisms

Takeshi Aizawa, Machiko Hatsumi* and Ken-Ichi Wakahama

Department of Biology, Faculty of Science, Shimane University
1060 Nishikawatsu-cho, Matsue 690, Japan

ABSTRACT—Isozyme polymorphisms of eight *Chaenogobius* species (Family Gobiidae) were studied in order to understand the phylogenetic relationships between them. Fifteen loci from ten enzymes and sarcoplasmic protein were detected by horizontal starch gel electrophoresis. The phylogenetic tree obtained from genetic distances between species essentially agreed with morphological and ecological studies previously reported. In addition, two new features were revealed. First, Nei's genetic distance between *C. laevis* from Saitama and that from Akita suggested that they are differentiate from each other on the species level. Second, the undescribed taxon, *C.* sp., from Lake Shinji was closely related to *C. laevis* from Akita and the genetic distance between them was 0.114. The smallest genetic distance between distinct species of *Chaenogobius* was 0.103 between *C. urotaenia* and *C. isaza* obtained in this study. This shows the possibility that *C.* sp. is the different species of *C. laevis*, from Akita.

INTRODUCTION

The family Gobiidae accomplished distinctive adaptive radiation. Each species adapted to various environments and has various life histories. This makes Gobiidae an excellent material for studying the mechanisms of evolution and speciation in Pisces. *Chaenogobius*, one genus of Gobiidae, consists of several species members that have various life histories, namely, species adapted in brackish water or fresh water, marine species, amphidromous species and land-locked species. The ecological and evolutionary genetic studies of this genus may especially supply us useful information on evolutionary process of adaptation of fishes.

The phylogenetic study on the members of this species has been carried out, but not completed, yet [23]. Takagi [31] demonstrated that *C.urotaenia* (Hilgendorf) and *C. castaneus* (O'Shaughnessy) are different species. Morphological and ecological studies showed three types of *C. urotaenia*, one lives in freshwater, another lives in brackish water and the other lives in the middlereach type, and now these types are recognized as distinct species and called *C.urotaenia* (Japanese name, Ukigori), *C.*sp.2 (Japanese name, Shimaukigori) and *C.*sp.1 (Japanese name, Sumiukigori), respectively [1, 7, 15, 31, 32]. Takagi [33] discriminated *C. laevis* (Steindachner) from *C. castaneus* based on its morphology. *Chaenogobius castaneus* has three pairs of pit organs connected by sensory canals and lives in brackish water, whereas *C. laevis* has no canal system and lives in freshwater. His study was followed by the description of new morph of *Chaenogobius* species (Japanese tentative name; Shinjiko-haze by Koshikawa [12]) from Lake Shinji in Japan [13]. This morph lives in brackish water of lower salt condensation than *C. castaneus* lives. It is similar to *C. laevis* for its color pattern and nuptial color of female but different from *C. laevis* and *C.castaneus* since shinjiko-haze has two pairs of pit organs connected by sensory canals on its head. This opening pattern of sensory canals is quite similar to *C. taranetzi* Pinchuk distributing Ussuri Bay in Russia and North Korea [9,23]. From these facts there are three possibilities of phylogenetic position of the species from Lake Shinji; namely, this species is one geographic variation of *C. laevis* or *C. taranetzi*, this is different species of *C. laevis* or *C. taranetzi*, and this species and *C. taranetzi* are the geographic variation of *C. laevis*.

In two decades of population biology, it was turned out that molecular approach is powerful to study phylogenetic relationships of various organisms [6, 18, 22, 27, 35]. Isozyme polymorphisms detected by electrophoresis supply useful measurement of genetic differentiation between populations or species, in terms of, the genetic distance [16, 17]. Many groups of species were studied on isozyme polymorphisms [24]. Accumulated data revealed that there were levels of genetic differentiation between local populations, subspecies, species or genus [6, 16, 17, 22, 24]. Using these levels, a phylogenetic tree could be constructed by cluster analysis from genetic distances [17, 2, 3, 4, 5]. For the species under consideration, electrophoretic studies should supply useful information on the phylogenetic relationships of *Chaenogobius* species.

In this study we analyzed allozyme polymorphisms of eight members of *Chaenogobius* species, six taxa mentioned above, *C. isaza* Tanaka, endemic to Lake Biwa in Japan, and *C. heptacanthus* (Hilgendorf), marine species, in order to clarify the phylogenetic relationships of this group and to characterize the species from Lake Shinji genetically.

Accepted May 10, 1994
Received November 20, 1994
* To whom all correspondence should be addressed.

MATERIALS AND METHODS

Animal sampling

Materials used in this experiment were *C. castaneus, C. laevis, C. heptacanthus, C. urotaenia, C.* sp. 1, *C.* sp. 2, *C. isaza* and an undescribed taxon collected from Lake Shinji , Shimane Prefecture in Japan. Since we could not conclude the undescribed taxon from Lake Shinji as *C. laevis* or *C. taranetzi*, we called this as *C.* sp. here. Table 1 shows the collection data of materials and Figure 1 shows the sites of collection. Six populations were kindly supplied by others, or, *C.* sp. 1 from Daitobetsu river by Dr. A. Goto, Faculty of Fisheries, Hokkaido University, *C. laevis* from Lake Hachiro-gata by Mr. K. Shibuya, Akita Prefectural Fisheries Consulting Center, *C. laevis* from Koma river by Mr. A. Iwata, Akasaka Imperial Palace, C. castaneus from Tama river by Mr. I. Kimoto, Tokyo Metropolitan Fisheries Experiment Station, *C. isaza* from Lake Biwa by Dr. S. Takahashi and Mr. S. Matsuoka, Shiga Prefecture and C. heptacanthus from Lake Nakaumi, Mawatashi by Mr. T. Kawashima, Mitoya Inland Water Fisheries Branch, Shimane Prefectural Fisheries Experiment Station.

Sample preparation for electrophoresis

Samples of fishes were stored at −25°C before dissection. Liver and lateral muscle were dissected out from each individual melted on ice. Three times or same amount of distilled water was added to liver or muscle, respectively, and homogenized in a microcentrifuge tube by plastic homogenizer on ice. The sample was centrifuged at 10000×*g* for 15 min. at 4°C. The supernatant was absorbed by capilalies and stored at −25°C until electrophoresis.

Individuals from which the tissues were removed were fixed in 10% formaldehyde. Identification of three species, *C. castaneus, C. laevis* and *C.* sp. was made by their color patterns after fixation and patterns of sensory canals, according to the method by Takagi [34].

Electrophoresis

Ten different enzymes and sarcoplasmic protein prepared from the species were analyzed by horizontal starch gel electrophoresis (Table 2). Two buffer systems were used in this experiment. One is citrate-aminopropyl morphorine buffer [8] and the other is citrate-tris buffer [21]. The staining methods of enzymes used were described by Shaw and Prasad [29] or Selander *et al.* [28]. Gels were dried between serophan to form films [20] and the isozyme patterns were documented on the films. When one enzyme had two loci, each locus was numbered in order of lower mobility to the anode.

Table 1. Collection data of materials

Species	P*	Locality, prefecture	Date	N**
C. castaneus	1.	Lake Nakaumi (Shimo-itou), Shimane	Dec. 1989	55
	2.	Iinashi River, Shimane	Nov. 1990	20
	3.	Lake Shinji (Matsue-Onsen), Shimane	Jan. 1990	20
	4.	Lake Shinji (Hamasada), Shimane	Jan. 1990	14
	5.	Lake Shinji (Tamayu), Shimane	Jan.-Feb. 1990	5
	6.	Tama River, Tokyo	Oct. 1989	3
C. sp.	7.	Lake Shinji (Matsue-Onsen), Shimane	Jan.-Feb. 1990	20
	8.	Lake Shinji (Hamasada), Shimane	Jan. 1990	21
	9.	Lake Shinji (Tamayu), Shimane	Jan.-Feb. 1990	34
C. laevis	10.	Lake Hachirou-gata, Akita	Dec. 1989	75
	11.	Koma River, Saitama	Oct. 1990	20
C. heptacanthus	12.	Lake Nakaumi (Mawatashi), Shimane	Dec. 1989	34
C. urotaenia	13.	Shinshi River, Shimane	Feb.-Mar. 1990	5
	14.	Satoji River, Shimane	Apr. Dec. 1990	4
	15.	Fukaura River, Shimane	Dec. 1990	2
	16.	Motoya River, Shimane	Dec. 1990	3
C. sp. 1	17.	Daitobetsu River, Hokkaido	Nov. 1990	20
C. sp. 2	18.	Shinshi River, Shimane	Feb.-Mar. 1990	13
	19.	Satoji River, Shimane	Apr. 1990	10
	20.	Fukaura River, Shimane	Dec. 1990	8
	21.	Ujiki River, Shimane	Dec. 1990	10
	22.	Oku River, Shimane	Jan. 1991	10
C. isaza	23.	Lake Biwa, Shiga	Jan.-Feb. 1991	45

P*; Population number.
N**; No. of individuals used for analysis.

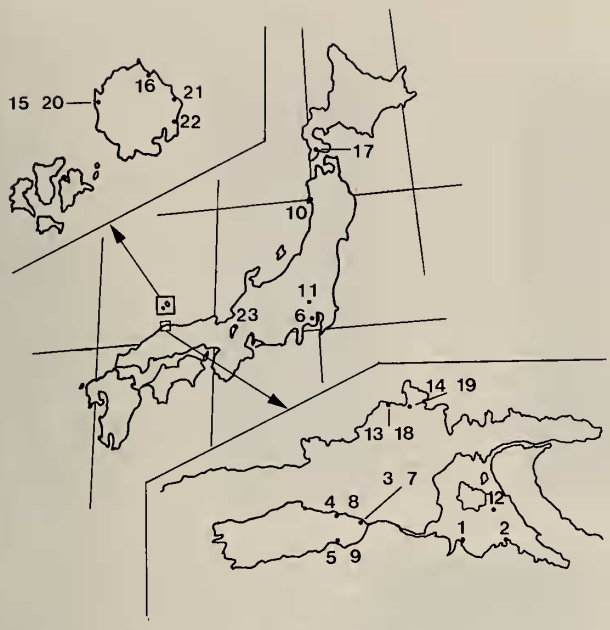

FIG. 1. The map of Japan showing sampling localities. Each number is corresponding to the population number in Table 1.

RESULTS

Fifteen loci were postulated in ten enzymes and sarcoplasmic protein from electrophoretic morph on starch gels among eight taxa of the genus *Chaenogobius*. Examples of zymograms were shown in Figure 2. Two types of bands were observed in one of the sarcoplasmic proteins. One type showed a single band and the other type showed double bands (Fig 2d, *sp-1*). Since each species had one of these types alternatively, double bands should not be heterozyotes and may be originated from gene duplication or post-translational modification. We named *b* to the allele of single band and *a* to the allele of double bands.

One locus, the *sarcoplasmic protein 2*, was monomorphic and the *Ldh* locus was also monomorphic with one exceptional individual from the Akita populations of *C. laevis* (Fig. 2b) but other loci had more than two alleles. Table 3 shows the allelic frequencies at thirteen loci on each population.

Each species had fixed allele at five loci, namely, *Aat, Ck, Me-1, Me-2* and *Sp-1*. In these loci *Ck, Me-1 Me-2* and *Sp-1* had only two variants. One type of variants was shared by *C. castaneus, C. sp., C. laevis, C. heptacanthus* and the other type was shared by *C. urotaenia, C.* sp. 1, *C.* sp.2 and *C. isaza*.

Other loci were polymorphic in some species. More than six alleles were observed at the *α-Gpd, Gpi-2, Mdh,* and *Pgm* loci. Twenty three populations were polymorphic for any of these four loci. Related species and populations were compared to each other for alleles of these four loci. At the *α-Gpd* locus, allele *f* was shared by *C.* sp. and the Akita population of *C.laevis* whereas the Saitama population of *C. laevis* had allele *d*. In *C. castaneus*, allele *e* of this locus was common. Two populations of *C. castaneus* had allele *b* at low frequencies while allele *b* was fixed in *C. heptacanthus* and *C.* sp. 1 and was at high frequency in *C. urotaenia. C.* sp.2 had allele *a* and *C. isaza* had allele *c* at the *α-Gpd* locus. At the *Gpi-2* locus, allele *c* was observed at high frequency in *C. castaneus*, *C.* sp. and *C. laevis* from Saitama while allele *b* was observed at high frequency in *C. laevis* from Akita and *C. heptacanthus*. *C. urotaenia, C.* sp.1 and *C.* sp. 2 had allele *d* at high frequency and allele *f* at low frequency. *C. isaza* had two alleles, *d* and *f*, at equal frequency at the *Gpi-2* locus. At the *Mdh* locus allele *e* and allele *f* were shared by *C. castaneus* and *C. laevis* but frequency was different in species. *C.* sp. had allele *e* and allele *a* but not allele *f*. *C. urotaenia, C.* sp. 1, *C.* sp. 2, *C. heptacanthus* and *C. Isaza* were monomorphic for the *Mdh* locus. At the *Pgm* locus allele *e* was common in *C. castaneus, C.* sp. and *C. laevis* from Akita whereas *C. laevis* from Saitama did not have allele *e* but had allele *c* and allele *b*. *C. urotaenia, C.* sp. 1, *C.* sp. 2 and *C. isaza* shared allele *c* at the *Pgm* locus at high frequency with other variants at low frequency. *C. heptacanthus* had allele *d* and allele *f* specifically.

The *Gpi-1* locus had two alleles, *a* and *b*. In almost all species, allele *b* was fixed. At the *Gpi-1* locus allele *a* was

TABLE 2. The list of Enzymes and proteins detected

Enzyme and protein (Abbreviation)	E.C.Number	Tissue*	Buffer**
Asparate aminotransferase (AAT)	2.6.1.1	M	CT
Creatine Kinase (CK)	2.7.3.2	M	CT
α-glycerophosphate dehydrogenase (α-GPD)	1.1.1.8	M	CT
Glucosephosphate isomerase (GPI)	5.3.1.9	M	CT
Isocitrate dehydrogenase (IDH)	1.1.1.42	M, L	APM
Lactate dehydrogenase (LDH)	1.1.1.27	M	APM
Malate dehydrogenase (MDH)	1.1.1.37	L	APM
Malic enzyme (ME)	1.1.1.40	M	CT
Phosphoglucomutase (PGM)	2.7.5.1	M	APM
Superoxide dismutase (SOD)	1.15.1.1	L	APM
Sarcoplasmic protein (SP)	—	M	APM

*; M means muscle and L means liver. **; AMP means citrate, aminopropyl mophorine buffer and CT means citrate-tris buffer.

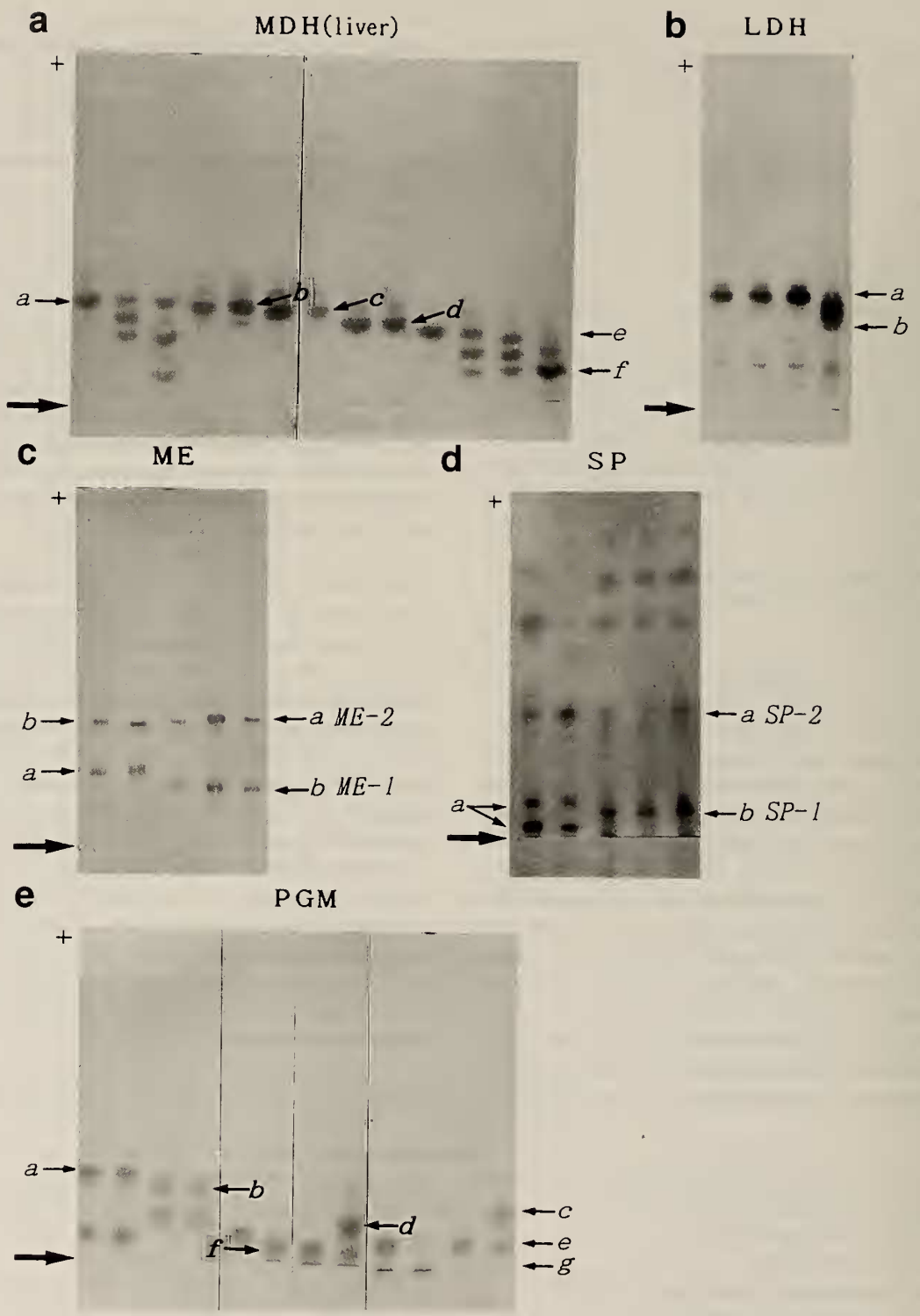

FIG. 2. Zymograms of four enzyme systems and a sacroplasmic protein. Thick arrows indicate origin.

only found in *C.* sp. and *C.* sp.1. The *Idh-1* locus had four alleles, the *Idh-2* had three alleles and the *Sod* had four alleles. These three loci, however, were not so polymorphic as other loci.

Nei's genetic distances [16] were calculated from the allele frequencies. Table 4 gives the matrix of average minimal and maximal genetic distances between each pair of species. The genetic distances between populations within species were 0 to 0.018 except 0.194 between the Akita and Saitama populations of *C. laevis*. This made us to list average genetic distances of each population of *C. laevis* separately. The genetic distances between species ranged from 0.092 between *C. urotaenia* and *C. isaza* to 1.595 between *C. heptacanthus* and *C.* sp. 2.

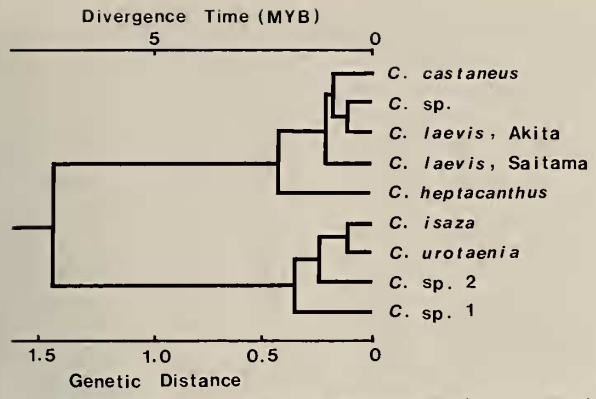

FIG. 3. Phylogenetic tree showing the relationships among eight species of genus *Chaenogobius* based on values of genetic distance.

A phylogenetic tree was constructed from genetic distances by average distance method (UPGMA) devised by Sneath and Sokal [30] and modified by Nei [17]. Figure 3 shows the phylogenetic tree of nine taxa of *Chaenogobius* constructed from average genetic distances in this study. The tree was not different topologically from the tree constructed from genetic distances between populations.

Eight species were divided into two groups. One was *C. castaneus* group including *C. castaneus, C.* sp. *C. laevis* and *C. heptacanthus* and the other was *C. urotaenia* group including *C. urotaenia, C.* sp. 1, *C.* sp. 2 and *C. isaza*. The genetic distances between two species from different groups were more than 1.2. In *C. castaneus* group *C. heptacanthus*, marine species, was separate from other three species, since the genetic distances between *C. heptacanthus* and other species in *C. castaneus* group were larger than 0.3. In *C. urotaenia* group, *C. urotaenia* and *C. isaza*, endemic to Lake Biwa, were closely related and the average genetic distance between them was 0.103.

Figure 3 shows the curious situation of *C. laevis*. The Akita population of *C. laevis* was differentiated from the Saitama population of *C. laevis* genetically. The Akita population was most closely related to *C.* sp. from Lake Shinji and closely related to *C. castaneus* more than the Saitama population of *C. laevis*.

DISCUSSION

In the above studies, it is seen that genetic variabilities in populations were low in *Chaenogobius* species. Expected average heterozygosity ranged from 0.006 in *C.* sp. 2. to 0.062 in *C.* isaza and polymorphic loci, from 0% in *C.* sp. 1 and *C.* sp. 2 to 26.7% in *C. castaneus* and *C. laevis* (Table 5). However these values are comparable to those in populations of Pisces species previously reported [19,24].

This study showed that the application of molecular taxonomy, based on isozyme polymorphisms, is useful to reveal the phylogenetic relationships among morphologically similar Gobiidae species as Masuda *et al.* [14] showed in their studies of *Rhinogobius* species.

The genus *Chaenogobius* was divided into two groups in this study (Fig. 3). One was *C. castaneus* group and the other was *C. urotaenia* group. One group shared the alleles different from the other group's at four loci, *Ck, Me-1, Me-2* and *Sp-1* (Table 3). The morphological studies have shown that *C. urotaenia, C.* sp.1, *C.* sp. 2 and *C. isaza* are similar to each other in its lateral line system and its large mouth when they were compared to other *Chaenogobius* species [1, 23]. Moreover the genetic distance between the two groups was more than 1.2 and this value was large enough to regard that they were genetically differentiated at genus level [18].

It was confirmed that three species of *C. castaneus* group, *C. castaneus, C.* sp. and *C. laevis*, morphologically similar each other, are closely related species. In addition, it was found that two *C. laevis* populations were highly differentiated. The Akita population of *C. laevis* was closely related to *C.* sp. and have more similar genetic population structure to *C. castaneus* than to the Saitama population of *C. laevis*. We could not find any difference in their sensory canals on their heads between individuals from Saitama and Akita of *C. laevis* although morphological differences between local populations of *C. laevis* were observed (Iwata, personal communication). In Lake Shinji, *C. castaneus* and *C.* sp. are sympatrically distributed and no hybrid individual was observed in this study. Because hybrid individuals should be easily detected, if they are, since different *α-Gpd* alleles were fixed in each species. This shows that there is no gene exchange between *C.castaneus* and *C.* sp. in Lake Shinji, which confirmed that *C. castaneus* and *C.* sp. are different species. This also suggests that *C. castaneus* and the Akita population of *C. laevis* are different species because the genetic distance between them was larger than that between *C. castaneus* and *C.* sp. And it is possible that the Akita population of *C. laevis* is different species of the Saitama population of *C. laevis* since the Saitama population situated on a different cluster from *C. castaneus, C.* sp. and *C. laevis* from Akita (Fig. 3). This is supported by the fact that Akita population was different from Saitama population in variants of *α-Gpd* and *Pgm* loci.

The genetic distance between *C.* sp. and the Akita population of *C. laevis* is 0.114. It is difficult to decide from this value whether *C.* sp. and the Akita population of *C. laevis* are different species or not. There are species whose genetic identity (I) between closely related species are about 0.9 [36] that corresponds to 0.105 of Nei's genetic distance. Furthermore *C.* sp. is different from *C. laevis* from Akita in patterns of sensory canals and pit organs on its head. These facts suggests the possibility that *C.* sp. and the Akita population of *C. laevis* are incipient species or subspecies.

The genetic distance between populations of *C. urotaenia* and *C. isaza*, sympatric species in Lake Biwa, was from 0.092 to 0.120. This is comparable to the genetic distance between *C.* sp. and the Akita population of *C. laevis* from 0.113 to 0.115. The studies on reproductive isolating mechanisms and/or ecological studies on these two species

TABLE 3. Allele frequencies at 14 loci in

Locus	Allele	*C. castaneus*						*C.* sp.			*C. laevis*		*C. heptacanthus*
		1	2	3	4	5	6	7	8	9	10	11	12
Aat	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—
	b	—	—	—	—	—	—	—	—	—	—	—	1.00
	c	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	—
Ck	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	—	—	—	—	—	—	—	—	—	—	—	—
Gpi-1	*a*	—	—	—	—	—	—	.03	—	—	—	—	—
	b	1.00	1.00	1.00	1.00	1.00	1.00	.97	1.00	1.00	1.00	1.00	1.00
Gpi-2	*a*	—	—	—	—	—	—	—	—	—	—	—	.09
	b	.01	—	—	—	—	—	—	—	—	.93	—	.85
	c	.98	1.00	.97	1.00	1.00	1.00	.97	1.00	1.00	.07	1.00	.06
	d	—	—	—	—	—	—	—	—	—	—	—	—
	e	.01	—	.03	—	—	—	.03	—	—	—	—	—
	f	—	—	—	—	—	—	—	—	—	—	—	—
α-Gpd	*a*	—	—	—	—	—	—	—	—	—	—	—	—
	b	.01	.03	—	—	—	—	—	—	—	—	—	1.00
	c	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	1.00	—
	e	.99	.97	1.00	1.00	1.00	1.00	—	—	—	—	—	—
	f	—	—	—	—	—	—	1.00	1.00	1.00	1.00	—	—
Idh-1	*a*	—	—	—	—	—	—	—	—	—	.03	—	—
	b	.16	.08	.08	—	.20	—	1.00	1.00	1.00	.88	1.00	1.00
	c	—	—	—	—	—	—	—	—	—	—	—	—
	d	.84	.92	.92	1.00	.80	1.00	—	—	—	.09	—	—
Idh-2	*a*	—	—	—	—	—	—	.03	.05	—	—	—	—
	b	1.00	1.00	1.00	1.00	1.00	1.00	.94	.95	1.00	1.00	1.00	—
	c	—	—	—	—	—	—	.03	—	—	—	—	—
Ldh	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.99	1.00	1.00
	b	—	—	—	—	—	—	—	—	—	.01	—	—
Mdh	*a*	—	—	.03	—	—	—	.18	.29	.22	.01	—	—
	b	—	—	—	—	—	—	—	—	—	—	—	—
	c	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	—
	e	.44	.45	.30	.36	.30	.50	.82	.71	.78	.10	—	—
	f	.56	.55	.67	.64	.70	.50	—	—	—	.89	1.00	1.00
Me-1	*a*	—	—	—	—	—	—	—	—	—	—	—	—
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Me-2	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	—	—	—	—	—	—	—	—	—	—	—	—
Pgm	*a*	.01	—	—	—	.10	—	—	—	—	—	—	—
	b	—	—	—	—	—	—	—	—	—	—	.28	—
	c	.05	.05	—	—	—	—	—	.02	—	—	.72	—
	d	—	—	—	—	—	—	—	—	—	—	—	.03
	e	.94	.95	1.00	1.00	.90	.83	1.00	.98	1.00	.81	—	—
	f	—	—	—	—	—	—	—	—	—	—	—	.97
	g	—	—	—	—	—	.17	—	—	—	.19	—	—
Sod	*a*	—	—	—	—	—	—	—	—	—	—	—	—
	b	—	—	—	—	—	—	—	—	—	—	—	—
	c	—	—	—	—	—	—	—	—	—	—	—	—
	d	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Sp-1	*a*	—	—	—	—	—	—	—	—	—	—	—	—
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00